CARDIAC AND PULMONARY REPLACEMENT

IMPROVED HYPOTHERMIC PRESERVATION OF RAT HEARTS BY FUROSEMIDE

The effect of furosemide, a blocker of the $Na^+/K^+/Cl^-$ cotransporter, on hypothermic preservation of rat hearts was studied with use of the Langendorff perfusion system and electron microscopy. Furosemide significantly improved the mechanical recovery and the coronary flow of the hearts preserved for 8 hours in St. Thomas' Hospital cardioplegic solution at a temperature of 4° C. Furosemide at the concentration of 100 μmol/L was found to have an optimal effect, whereas at high concentrations (1000 μmol/L) it was found to have toxic effects. In addition, furosemide reduces the time elapsed between the end of the preservation time and the resumption of myocardial contractions. Ultrastructural evaluations were done in which the presence of swollen mitochondria was chosen as a criterion of hypothermic ischemic damage to the myocardium. Morphometric analysis indicated that the mitochondrial volume of hearts stored for 8 hours in St. Thomas' Hospital cardioplegic solution increased by 72% as compared with the mitochondrial volume of hearts that were not exposed to the hypothermic ischemic conditions (control group). The addition of 100 μmol/L furosemide to the cardioplegic solution resulted in a significant reduction of mitochondrial swelling during the period of 8 hours' storage, which amounted only to 28% as compared with the figure for the control group. The reduction of mitochondrial swelling by furosemide and the improved mechanical and coronary flow recoveries are thought to be related to the blocking of the sarcolemmal $Na^+/K^+/Cl^-$ cotransporter and consequently the reduction of the Na^+ influx during hypothermic ischemic storage. (J THORAC CARDIOVASC SURG 1995;110:523-31)

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Heart transplantation is a successful treatment for irreversible, final stage cardiac failure. However, despite intensive efforts that have been conducted toward extending heart preservation, to date, the time allowed for cold ischemic preservation of the heart is limited to 4 to 5 hours because reliable recovery of the heart cannot otherwise be achieved.[1] The most widely used procedure for heart preservation is based on a single infusion of a cold crystalloid cardioplegic solution, followed by hypothermic storage. The crystalloid cardioplegic solutions are made up of either extracellular-like formulation, such as St. Thomas' Hospital cardioplegic solution (ST), or as intracellular-like formulation, such as University of Wisconsin cold storage solution.

During hypothermic ischemic storage the intracellular ionic homeostasis is disturbed. The most drastic change is a large influx of Na^+,[2] followed by influx of water and an increase in cell volume.[3]

Mitochondrial damage is one of the main factors that accounts for the depression of cardiac function. Scanning electron microscopy of the ischemic heart

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shows derangement of individual cells because of the presence of interstitial edema, dissociation of myofibrils, loss of myofilaments, and swollen mitochondria with various degrees of cristae disorientation.[4-7]

In a previous study we demonstrated, with ^{23}Na nuclear magnetic resonance techniques, that loop diuretic agents, such as furosemide and bumetanide, can reduce the Na^+ influx during hypothermic heart storage when added to Krebs-Henseleit solution.[8] It was suggested that by blocking the $Na^+/K^+/Cl^-$ cotransporter located in the sarcolemma the accumulation of intracellular Na^+ is decreased, leading to protection of the myocardial cells during hypothermic ischemia.

In this study, we investigated the hemodynamic effects of furosemide on heart recovery after 8 hours of preservation in ST cardioplegic solution at 4° C. In addition, we conducted an electron microscopy study that measured the changes in myocardial mitochondrial volumes after 8 hours of hypothermic preservation in ST cardioplegic solution.

Material and methods

Hemodynamic study. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). Male Sprague-Dawley rats weighing 300 to 400 gm were randomly divided into two experimental groups. They were anesthetized with phenobarbital sodium (20 mg per rat intraperitoneally), and the hearts were excised and placed immediately in iced heparinized saline solution. After cessation of contractions, the aortas were cannulated for Langendorff perfusion. Ventricular pressure was measured by a thin latex balloon, which was inserted into the left ventricle across the mitral valve. The balloon was inflated with aqueous solution to achieve an initial end-diastolic pressure of 10 to 15 mm Hg and was then kept isovolumic throughout the experiment. This balloon was connected by a polyethylene tube to a pressure transducer that transmitted the pressure signals to an IBM AT-286 computer (IBM Corp., Armonk, N.Y.) that analyzed and computed the intraventricular pressure and its derivative (dP/dt). Immediately on connection to the perfusion system the hearts were paced (Harvard stimulator, Harvard Apparatus, S. Natick, Mass.) at a rate of 300 beats/min. Coronary flow was measured by collecting the effluent liquid that came out of the heart into a measuring vessel.

The experimental hearts were divided into six groups that consisted of five groups that were treated with furosemide, each with a different concentration (10, 50, 100, 300, and 1000 μmol/L), and of one control group that was not treated with furosemide. In the groups treated with furosemide the experimental protocol was as follows. Fifteen minutes of perfusion with a phosphate-free Krebs-Henseleit (KH) buffer solution at a temperature of 37° C and in a pressure of 95 cm of water was followed by 15 minutes of perfusion with KH solution with furosemide (Sigma Chemical Co., St. Louis, Mo.). Left ventricular pressure, dP/dt, and coronary flow were measured every 5 minutes. After perfusion for 30 minutes at 37° C, the hearts were perfused for 2 minutes with ST solution plus furosemide at a temperature of 4° C. In this 2-minute period the heart contractions were stopped. Then the hearts were placed in a 250 ml flask containing the same ST plus furosemide cardioplegic solution. This solution was kept cooled at a temperature of 4° C throughout 8 hours of preservation.

At the end of the preservation time, the hearts were reconnected to the Langendorff perfusion system and perfusion with KH buffer solution at a temperature of 37° C was commenced. The hearts were paced initially at a rate of 180 beats/min for 10 minutes, then at a rate of 300 beats/min for 20 minutes. The overall reperfusion time was 30 minutes, during which measurements of left ventricular pressure, dP/dt, and coronary flow were taken in cycles of every 5 minutes. The control group underwent the same protocol, but without furosemide.

The KH solution contained the following components: 121 mmol/L NaCl, 5.9 mmol/L KCl, 1.75 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 23 mmol/L $NaHCO_3$, and 11 mmol/L glucose. The solution was bubbled continuously with a mixture of 95% oxygen and 5% carbon dioxide, and the pH was 7.4. The ST cardioplegic solution contained the following components: 110 mmol/L NaCl, 16 mmol/L KCl, 16 mmol/L $MgCl_2$, 1.2 mmol/L $CaCl_2$, and 10 mmol/L $NaHCO_3$, and the pH was 7.8.[9]

Electron microscopy study. Male Sprague-Dawley rats weighing 300 to 400 gm were randomized into three experimental groups. Rats in group 1 (8 hours, control; 8h/c) were anesthetized with phenobarbital sodium (20 mg per rat intraperitoneally). Hearts were excised and placed immediately in iced heparinized saline solution. After contractions ceased, the aortas were cannulated for Langendorff perfusion. These hearts were perfused initially with KH solution at a pressure of 95 cm of water for 20 minutes, followed by 2 minutes of perfusion with the cold (4° C) ST solution.[9] Then the hearts were placed in a beaker containing cold ST solution and stored for 8 hours. At the end of the storage period the hearts were perfused with phosphate-buffered saline solution containing 2% glutaraldehyde for 3 minutes. A cubic (0.5 cm^3) section of myocardial tissue was removed via a cut beneath the left auricle. This myocardial tissue was then cut into smaller cubes (1 mm^3) and placed in the glutaraldehyde solution for fixation.

Rats in group 2 (8 hours, furosemide treatment; 8h/f) underwent the same procedure but with the following variations. The preischemic 20 minutes of perfusion was divided into 5 minutes of perfusion with KH solution only, followed by 15 minutes of perfusion with KH solution containing 100 μmol/L furosemide. The ST solution in this group contained 100 μmol/L furosemide as well.

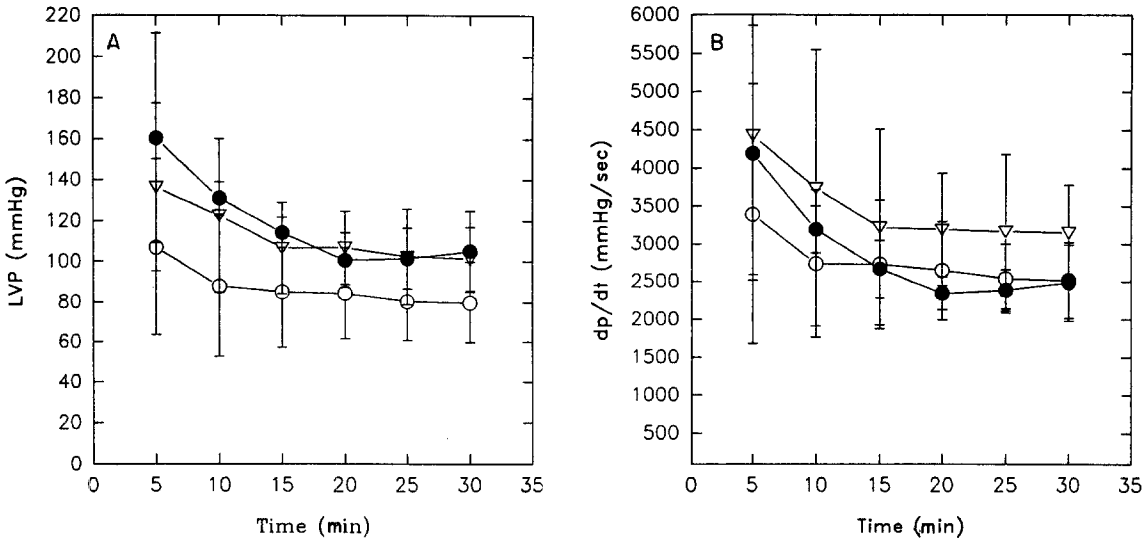

Fig. 1. A, Left ventricular pressure *(LVP)* at preischemic phase. **B,** dP/dt of left ventricle at preischemic phase. *Open circles* represent control group, *filled circles* represent group treated with 50 μmol/L furosemide, and *triangles* represent group treated with 100 μmol/L furosemide. Results are expressed as mean plus or minus standard deviation, $n = 5$.

Rat hearts in group 3 (not exposed to hypothermic ischemia; 0h/c) were cannulated on the Langendorff perfusion system as described and perfused with KH solution only for 20 minutes. After this period, the hearts were perfused immediately with glutaraldehyde followed by removal of the myocardial tissue as described for the other groups.

For electron microscopy, the 1 mm^3 blocks of glutaraldehyde-fixed myocardium were washed with phosphate-buffered saline solution, dehydrated in ethanol, post-fixed with 1% OsO_4 in barbital (Veronal)-acetate buffer, pH 7.4, for 1 hour at 4° C, dehydrated in ethanol and propylene oxide, and embedded in araldite.

Morphometry. Analysis of mitochondrial size was done on electron micrographs of thin araldite sections at a magnification of ×25,000. The perimeter and area of the mitochondria were measured by a MOP-Vidioplan system (Kontron, Munich, Germany) with the standard program. For each heart sample, the measurements were done on 100 different mitochondria, arbitrarily selected from five micrographs of different sections representing different areas of the tissue block. To rule out the possibility of irregularity of mitochondrial shape, which may affect the correlation of area to volume, we also calculated the form factor of each sample, as defined by the correlation

$$\text{Form factor} = \frac{4\pi \times \text{area}}{\text{Perimeter}^2}$$

In all samples measured the form factors were similar (0.75 ± 0.08), indicating that direct correlation of area to volume can be made.

Statistical analysis. Results are expressed as the mean plus or minus the standard deviation. Statistical significance was tested by one-way analysis of variance, with the multiple range test Scheffe procedure, and Student's *t* test for paired observations. A value of $p < 0.05$ was considered to indicate a significant difference.[10]

Results

Hemodynamic study. The preischemic effect of furosemide on left ventricular pressure and dP/dt are summarized in Fig. 1. The results indicate no significant difference between the control group and the groups treated with 50 μmol/L furosemide or with 100 μmol/L furosemide. However, the results obtained after 8 hours of preservation in the ST cardioplegic solution indicated that the group treated with 100 μmol/L furosemide achieved a significantly higher level of mechanical recovery during the 30 minutes of reperfusion as shown in Fig. 2. Despite the positive mechanical effect of furosemide that was found in the group treated with 50 μmol/L furosemide, the results in this group did not achieve statistical significance compared with those in the control group.

Fig. 3 summarizes the percentage of mechanical recovery after 8 hours of preservation in the ST cardioplegic solution. These results were obtained by calculating the ratio between the values obtained after 15 minutes of perfusion in the preischemic phase (before furosemide administration) and the values obtained at the end of the 30 minutes of reperfusion (after the end of the preservation time).

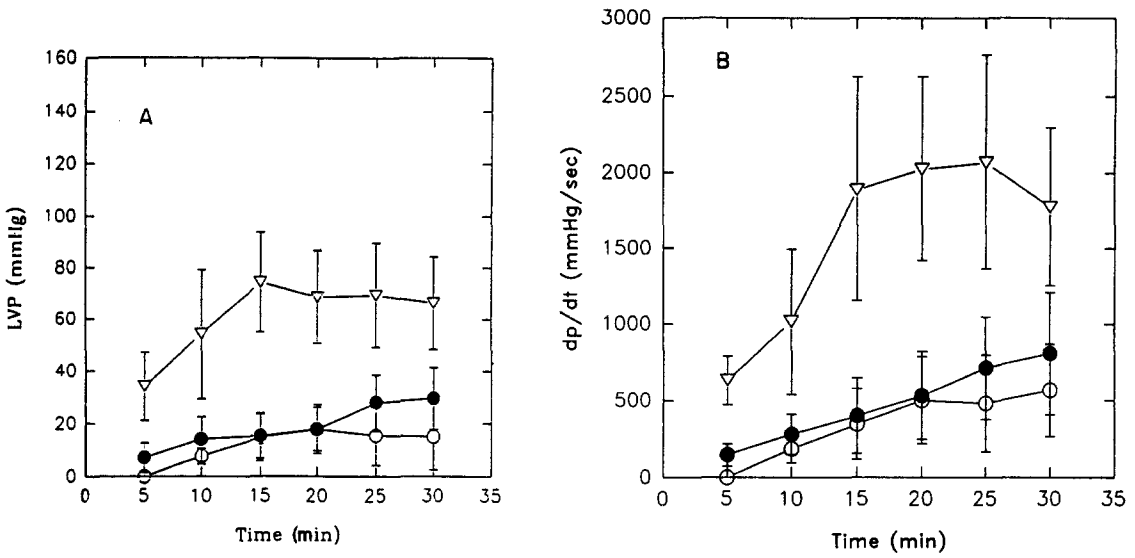

Fig. 2. Recovery during 30 minutes of reperfusion, started after 8 hours of preservation in ST cardioplegic solution. **A,** Left ventricular pressure *(LVP)* recovery. **B,** dP/dt recovery. *Open circles* represent control group, *filled circles* represent group treated with 50 μmol/L furosemide, and *triangles* represent group treated with 100 μmol/L furosemide. Results are expressed as mean plus or minus standard deviation, $n = 5$.

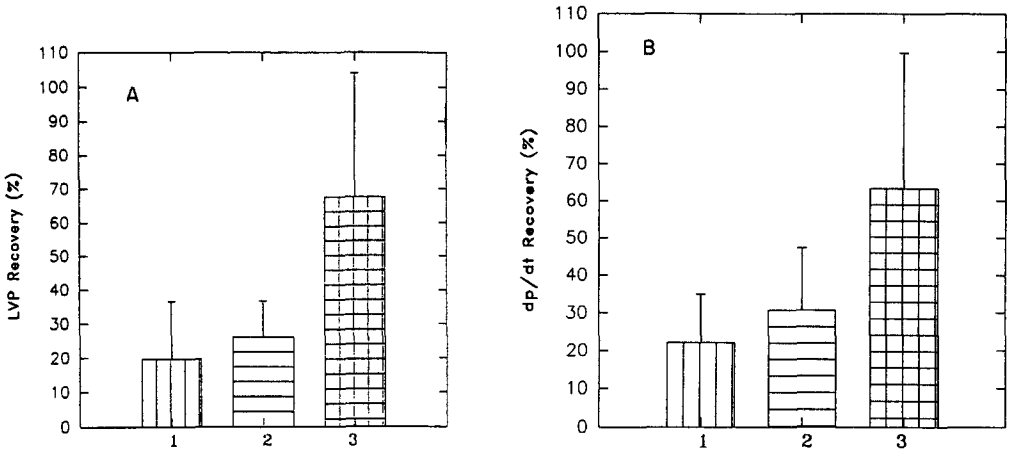

Fig. 3. Recovery percentage after 8 hours of preservation in ST cardioplegic solution. Percentage was calculated from ratio between preischemic values (after 15 minutes of perfusion) and values at end of 30 minutes of reperfusion. **A,** Left ventricular pressure *(LVP)* recovery percentage. **B,** dP/dt recovery percentage. *1,* Control group; *2,* group treated with 50 μmol/L furosemide; *3,* group treated with 100 μmol/L furosemide.

It is clearly shown in Fig. 3 that the treatment with 100 μmol/L furosemide improved drastically the mechanical recovery. An important parameter was obtained by calculating the time that elapsed until the hearts showed mechanical work after the 8 hours of preservation. None of the hearts in the control group showed mechanical work within the first 5 to 10 minutes of reperfusion. However, all the hearts in the treated groups (50 μmol/L and 100 μmol/L furosemide) showed mechanical work within the first 2 minutes of reperfusion.

The effects of furosemide on left ventricular pressure and dP/dt were tested in three additional concentrations: 10 μmol/L, 300 μmol/L, and 1000 μmol/L. Furosemide concentrations of 10 μmol/L and 300 μmol/L were found to have no significant

Table I. *Preischemic coronary flow**

Time (min)	*Control (ml/min)*	*Furosemide (ml/min)*	
		50 μmol/L	*100 μmol/L*
5	17.1 ± 3.4	17.0 ± 2.6	19.2 ± 2.3
10	17.6 ± 3.4	16.7 ± 2.3	18.8 ± 1.8
15	17.4 ± 3.8	16.2 ± 2.3	18.8 ± 1.8
20	17.7 ± 4.9	16.5 ± 2.5	20.8 ± 1.8
25	17.1 ± 5.3	17.0 ± 2.6	21.4 ± 1.9
30	17.1 ± 5.3	17.5 ± 2.5	21.4 ± 1.9

*The coronary flow as a function of the time elapsed from mounting of the hearts on the Langendorff perfusion system, $n = 5$.

Table II. *Postischemic coronary flow**

Time (min)	*Control (ml/min)*	*Furosemide (ml/min)*	
		50 μmol/L	*100 μmol/L*
5	—	7.7 ± 1.3	14.0 ± 3.2
10	10.1 ± 1.9	7.7 ± 1.3	14.4 ± 3.8
15	9.7 ± 2.1	8.7 ± 1.5	14.2 ± 3.4
20	9.7 ± 1.9	8.7 ± 1.5	14.2 ± 3.7
25	9.7 ± 1.9	9.2 ± 1.0	13.8 ± 3.0
30	9.1 ± 1.6	9.0 ± 0.8	13.4 ± 3.3

*The coronary flow after 8 hours of preservation in ST solution measured as a function of the time elapsed from the onset of reperfusion, $n = 5$.

effect on the mechanical recovery of the hearts. However, the hearts treated with these two concentrations also resumed mechanical work after the 8 hours of preservation within the first 2 minutes of reperfusion. Furosemide at the concentration of 1000 μmol/L was found to have toxic effect. Hearts treated with this concentration showed no recovery at all.

Tables I and II summarize the coronary flow results obtained during perfusion at the preischemic phase and during reperfusion at the postischemic phase. These results indicate no significant difference between the control and the treated groups during perfusion in the preischemic phase. However, it is important to note that in the group treated with 100 μmol/L furosemide, a significant difference was found between the values obtained at the end of 15 minutes of perfusion and the values obtained after 30 minutes of perfusion (that is, 15 minutes after the onset of furosemide administration). The coronary flow results during reperfusion after the 8 hours of preservation in ST solution indicate no significant difference between the control group and the group treated with 50 μmol/L furosemide. However, the coronary flow in the group treated with 100 μmol/L furosemide was significantly higher than that in the control group after the 8 hours of preservation.

Electron microscopy study. Swollen mitochondria were chosen as a primary criteria of hypothermic ischemic damage in the myocardium. Table III summarizes the area of the mitochondrial section of hearts in each experimental group. We measured 100 mitochondria per heart. Eight hours of storage in the ST cardioplegic solution for the 8h/c hearts resulted in a considerable increase in mitochondrial volume as compared with that in the immediately fixed control 0h/c hearts (Fig. 4). The mitochondria in the 8h/c hearts (Fig. 5) were highly dilated and exhibited considerably larger average section areas

Table III. *Mitochondrial average section areas**

Heart	*Area (μm^2)*		
	8h/c	*8h/f*	*0h/c*
1	0.62 ± 0.39	0.43 ± 0.29	0.31 ± 0.21
2	0.50 ± 0.31	0.34 ± 0.24	0.28 ± 0.17
3	0.44 ± 0.27	0.34 ± 0.16	0.36 ± 0.25
4	0.46 ± 0.32	0.34 ± 0.19	0.22 ± 0.14
5	0.53 ± 0.32	0.46 ± 0.27	0.31 ± 0.24

*Mitochondrial average section areas of five hearts in each experimental group. The measurements were done on 100 mitochondria per heart. The results represent variation within the hearts in each group, as well as within mitochondria in each heart. Hearts in the 8h/c group underwent 8 hours of hypothermic preservation with ST only. Hearts in the 8h/f group underwent 8 hours of hypothermic preservation with ST plus 100 μmol/L furosemide. In the 0h/c group, hearts were not exposed to hypothermic preservation. Significance was tested by one-way analysis of variance with multiple range test indicating $p < 0.0001$.

as compared with those of the 0h/c hearts. Morphometric analysis indicated that the average section area of the mitochondria in the 8h/c hearts increased by 72% as compared with that in the 0h/c hearts. Hypothermic ischemic storage of hearts in the ST cardioplegic solution containing 100 μmol/L furosemide (8h/f) resulted in considerably improved preservation of the myocardium as indicated by the morphologic findings of the mitochondria (Fig. 6). This concentration of furosemide was selected because it gave a good inhibitory effect of the Na influx and an optimal mechanical recovery.[8] In most of these myocardia, the mitochondria were almost identical to those found in the 0h/c hearts in shape and section area sizes. Morphometric analysis revealed that the increase in the average section area of the mitochondria in the 8h/f hearts was only 28% as compared with that of the 0h/c hearts (Fig. 7).

Discussion

In this study we demonstrated that furosemide, preischemically introduced and added to the ST cardioplegic solution, protects the myocardium of the heart during an 8-hour period of preservation.

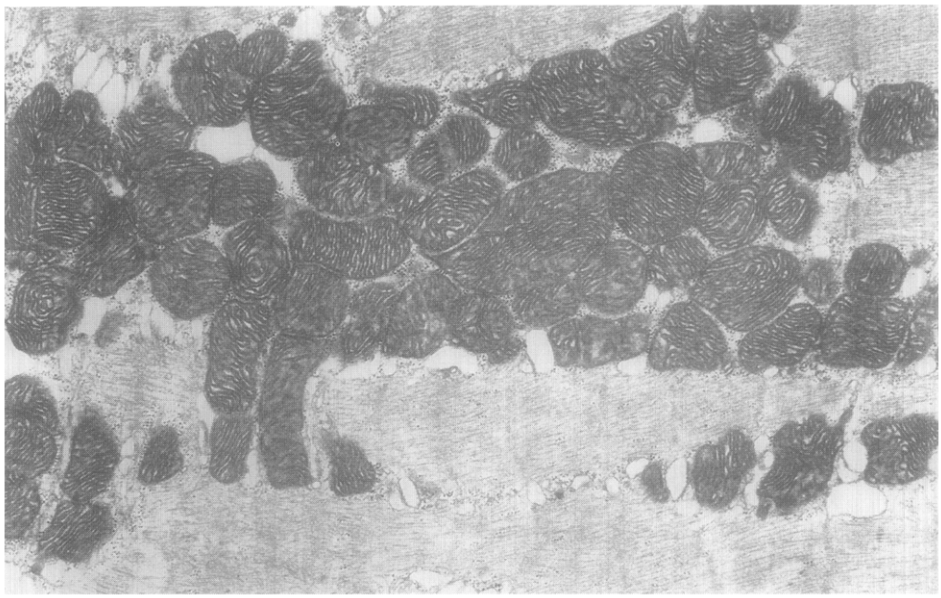

Fig. 4. Electron micrograph of araldite myocardial section of rat heart that was not exposed to hypothermic ischemic conditions, fixed immediately after excision (original magnification ×45,000).

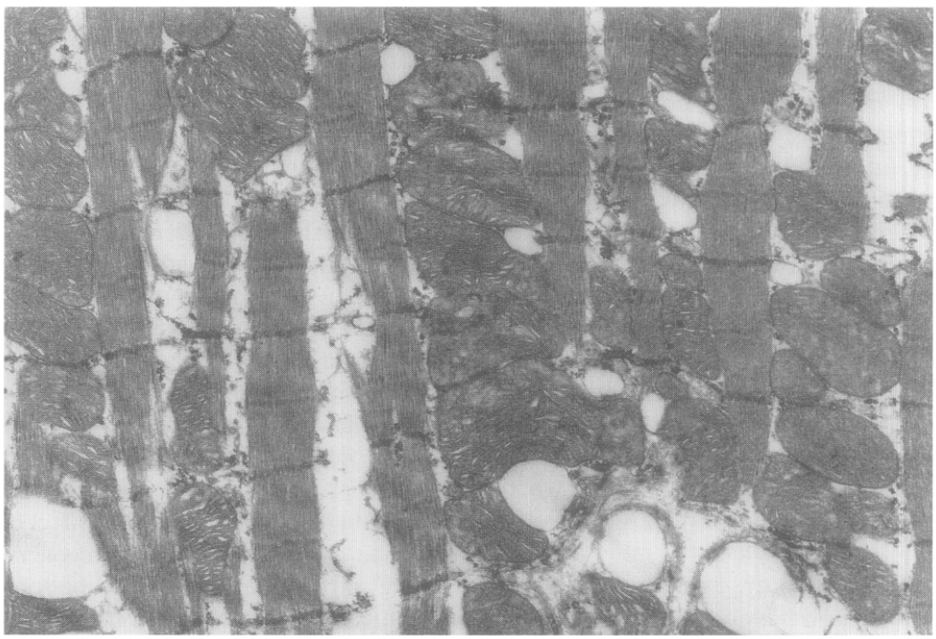

Fig. 5. Electron micrograph of araldite myocardial section of rat heart fixed after 8 hours of hypothermic ischemic preservation with ST solution. Note increase in size and decrease in density of mitochondria as compared with those in Fig. 4 (original magnification ×45,000).

This protection was indicated by the density, shape, and morphometry of the mitochondria and by improved mechanical recovery and coronary flow. In an earlier study we demonstrated that loop diuretics such as furosemide and bumetanide reduced the large increase in intracellular Na^+ accumulation during hypothermic ischemic preservation with KH.[8] This reduction in the intracellular accumulation of Na^+ is believed to be a result of blocking of the loop diuretic-sensitive $Na^+/K^+/Cl^-$ cotrans-

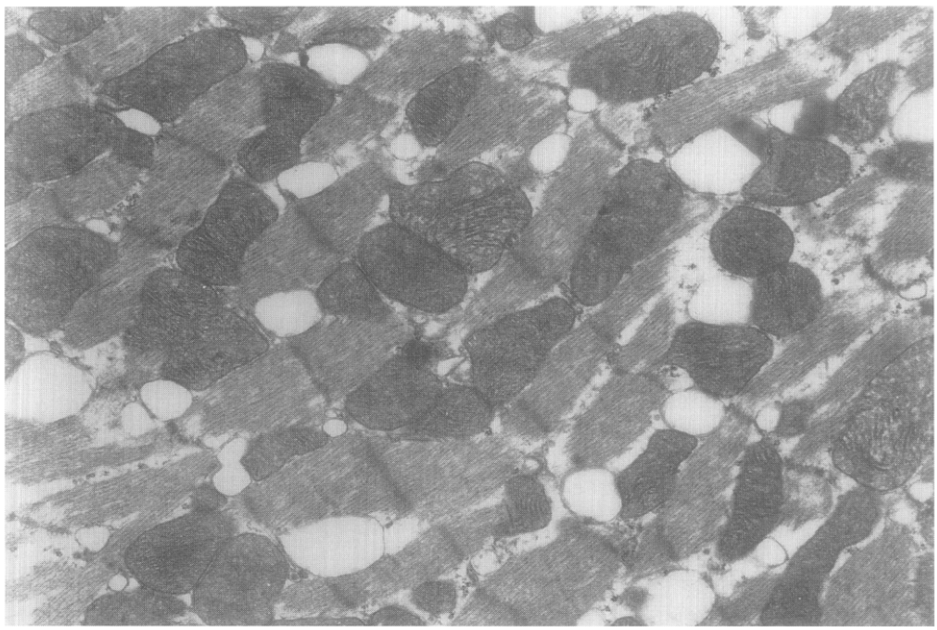

Fig. 6. Electron micrograph of araldite myocardial section of rat heart fixed after 8 hours of preservation with ST containing 100 μmol/L furosemide. Note resemblance of mitochondrial size and density to those of mitochondria in Fig. 4 (original magnification ×45,000).

porter, located in the myocardial sarcolemma.[11] Our hypothesis was that under hypothermic ischemic conditions Na/K adenosinetriphosphatase is no longer active, which causes a large accumulation of Na^+ inside the myocardial cells. Under the same conditions the $Na^+/K^+/Cl^-$ cotransporter, which operates passively, without using adenosine triphosphate hydrolysis for energy supply, plays a major role in the influx of Na^+.[12-14] As a consequence of the intracellular Na^+ accumulation, large amounts of water penetrate the myocardial cell, causing the cell to swell.[3] Because mitochondrial membranes are readily permeable to water, the accumulated water in the cytosol may enter into the mitochondrial matrix, causing mitochondrial swelling followed by disorientation of the cristae. Blocking the $Na^+/K^+/Cl^-$ cotransporter by furosemide reduces the intracellular Na^+ accumulation, as indicated in our previous study,[8] and as a consequence reduces intracellular and intramitochondrial water accumulation. This was indicated in this study by measurement of mitochondrial volumes. These effects are expected to improve the functional recovery of the heart after hypothermic preservation, as was indeed found experimentally in this study.

Furosemide in the concentration of 100 μmol/L was found to have the most potent effect on both the mechanical recovery and the coronary flow. Although the results of our previous ^{23}Na nuclear magnetic resonance study indicated that furosemide at a concentration as low as 10 μmol/L achieved maximal reduction in the intracellular Na^+ accumulation in hearts stored in KH solution, this concentration was found to have no significant effect on the mechanical recovery of the hearts stored in ST cardioplegic solution. Thus the concentration range for the Na^+ transport inhibition may be different between the two preservation solutions or the effect of furosemide on the mechanical recovery involves other factors besides the intracellular Na^+ accumulation, such as its effects on the coronary flow. It is important to note that measurement of the intracellular Na^+ concentration in hearts preserved in ST cardioplegic solution is difficult because of the high concentration of K^+ in this solution. A high concentration of K^+ reduces the Dy-triethylenetetramine-hexaacetic acid–induced chemical shift between the extracellular and intracellular compartments, abolishing the ability to discriminate between these two compartments.

Furosemide at a high concentration (1000 μmol/L) was found to have toxic effects on the myocardium. This evidence agrees with findings of previous studies that indicated that high doses of furosemide

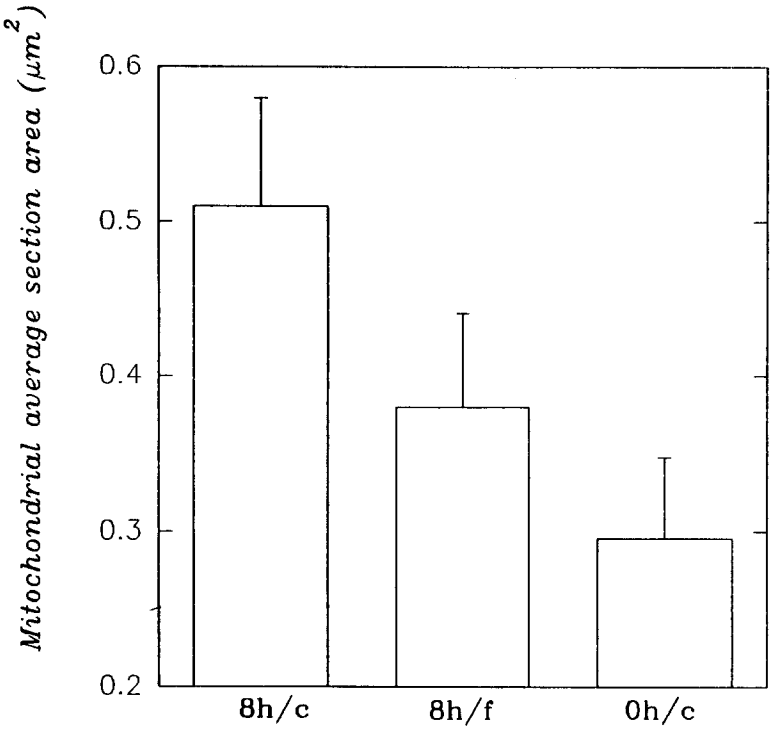

Fig. 7. Comparison between means of mitochondrial average section area of each group. Hearts in 8h/c group underwent 8 hours of hypothermic preservation with ST solution only. Hearts in 8h/f group underwent 8 hours of hypothermic preservation with ST solution plus 100 μmol/L furosemide. In the 0h/c group hearts were not exposed to hypothermic preservation. Significance was tested by Student's *t* test, which indicated a value of $p < 0.01$ between 8h/c and 8h/f groups, whereas the significance between 0h/c and 8h/f was $p < 0.05$.

markedly reduced rat heart recovery, even after 25 minutes of ischemia.[15] Furosemide reduces the time elapsed between the end of ischemic and hypothermic preservation and the resumption of myocardial contraction on reperfusion. This phenomenon can be explained by the time required by the myocardial cells to reestablish ionic homeostasis on both sides of the sarcolemma. In the treated groups, lower intracellular concentrations of Na^+ brought shorter elapsed times until the resumption of myocardial contraction. Administration of 100 μmol/L furosemide in the preischemic phase caused a significant elevation in the coronary flow. This finding agrees with the previous observations that indicated that furosemide increases coronary flow in a dose-dependent manner.[15] Because furosemide had no inotropic effect during the preischemic phase, it can be assumed that the elevation in the coronary flow was caused by a vasodilative effect of furosemide on the coronary endothelial cells. Recently, it was documented in the literature that administration of furosemide caused an increase of the synthesis of prostaglandin E_2 in the kidney.[16] Moreover, direct correlation was found between the elevation of prostaglandin E_2 concentration and the degree of vasodilative effect on the kidney's blood vessels.[17] This vasodilative effect of furosemide was blocked when indomethacin, known as a prostaglandin synthesis blocker, was used.[17] The prostaglandins I_2 and E_2 are known to have very potent vasodilative effects on the cardiovascular system. In studies done in dogs it was shown that the administration of these prostaglandins brought about a sharp reduction in arterial blood pressures.[18, 19] Thus it can be postulated that the effect of furosemide on coronary flow is caused by an increase in the synthesis and release of prostaglandins from the coronary endothelial cells, which in turn vasodilate the coronary blood vessels.

Furosemide is a loop diuretic agent commonly used in the treatment of patients with hypertension. In the present work, we have demonstrated its beneficial effect on the recovery of rat hearts preserved in ST cardioplegic solution under hypother-

mic conditions. Although it has been reported that University of Wisconsin solution gives improved preservation results when compared with ST solution,[20] we used the ST solution because of its wide use in clinical settings and because it is relatively simple to prepare. We believe that furosemide protects the myocardial tissue during hypothermic ischemic preservation by reducing the intracellular Na^+ accumulation, which in turn suppresses the elevation of the intracellular free calcium via the Ca^{2+}/Na^+ exchanger and reduces intracellular water accumulation as reflected by the reduction in mitochondrial swelling and damage, improved mechanical recovery, and increased coronary flow. If furosemide is found to be effective in human hearts as well, it would be easy to introduce this agent to clinical use in heart preservation programs.

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